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Effect of temperature on energetic demands during the last stages of embryonic development and early life of *Octopus vulgaris* (Cuvier, 1797) paralarvae

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Abstract

This study describes the effect of seasonal average temperatures (14 and 18°C) in the Ría of Vigo, on the utilization of external yolk over the last five Naef stages of development (XV–XX) for *Octopus vulgaris* embryos. Also, the transference of the outer yolk to the inner yolk sac, and its use during embryonic development and early life by *O. vulgaris* paralarvae. Temperature had a marked effect on embryonic development, except during stages XV–XIX (until the second inversion) where development time was the same (14 days), regardless of temperature. There were no significant differences in outer yolk decrease between consecutive Naef stages at 14°C and 18°C. Contrary, significant differences at all Naef stages from XV to XIX (both, with or without outer yolk) were observed for inner yolk between temperatures. A higher accumulation of inner yolk in embryos at 14°C was observed, due to lower yolk consumption. Paralarvae incubated at both temperatures were maintained independently at starvation during 4 days. At 18°C, a reduced accumulation of inner yolk, especially during Naef stage XIX, was observed. In 24 h old paralarvae, there was already significant higher inner yolk content at 14°C than at 18°C. Unfed paralarvae at 18°C lost weight faster than those at 14°C, due to higher energetic requirements. Finally, from these results, we propose a paralarvae rearing protocol during the first days after hatching and during the last five Naef stage (XV–XX) at lower temperatures, since the energy requirements are lower during the initial maturation stage.

Keywords: embryonic development, *Octopus vulgaris*, paralarvae, yolk reserves

Introduction

Octopus vulgaris (Cuvier 1797), is one of the most promising cephalopods for aquaculture diversification (Iglesias, Sánchez, Bersano, Carrasco, Dhont, Fuentes, Linares, Muñoz, Okumura, Roo, Van der Meeren, Vidal & Villanueva 2007; Iglesias & Fuentes 2014), since it has fast growth rates, food conversions, protein content, and a short life cycle (1–2 years). *O. vulgaris* was described as a cosmopolitan species (Mangold 1983), but taxonomy of this genus remained still unresolved. Moreover, based on genetic markers and morphological characteristics several authors establish five groups (*O. vulgaris sensu stricto*, type I, II, III and IV) within the classical definition of *Octopus vulgaris* Cuvier (1797) reviewed in Norman, Frederick, Hochberg and Finn (2013). Cabranes, Fernandez-Rueda and Martínez (2008) support the hypothesis of the existence of *O. vulgaris* intraspecific differences among populations within the group *O. vulgaris sensu stricto*. Such advances strongly indicate that adaptation to the environment conditions, such as temperature range, either during embryonic development or in the early planktonic stages (Repolho, Baptista, Pimentel, Dionısio, Trübenbach, Lopes, Lopes, Calado, Diniz & Rosa 2014), may be strongly associated to local condition than it was previously assumed.

The effect of temperature on the embryonic development of this species is well documented

(Boletzky 1987). However, with the exception of *Illex illecebrosus* (O'Dor, Foy, Helm & Balch 1986), *Sepia officinalis* (Bouchaud 1991), *Loligo opalescens* (Vidal, Di Marco, Wormuth & Lee 2002), *Loligo vulgaris* (Vidal, Roberts & Martins 2005) and *Octopus mimus* (Uriarte, Espinoza, Herrera, Zuñiga, Olivares, Carbonell, Pino, Farías & Rosas 2012), there are no published data on the effect of temperature on survival and rate of yolk utilization in cephalopod hatchlings. For *S. officinalis* (Boucher-Rodoni, Boucaud-Camou & Mangold 1987), *Loligo opalescens* (Vidal *et al.* 2002) and *O. vulgaris* (Villanueva, Koueta, Riba & Boucaud-Camou 2002; Iglesias, Fuentes, Sánchez, Otero, Moxica & Lago 2006), hatchlings start feeding before its yolk reserves are completely depleted, combining endogenous (yolk) with exogenous (prey) feeding.

Embryonic development of *O. vulgaris* has been described in the literature (Naef 1928; Mangold & Boletzky 1973; Iglesias, Otero, Moxica, Fuentes & Sánchez 2004). Naef (1923, 1928) described 20 different stages of embryonic development, classified according to morphological characteristics. The inner yolk sac becomes full in the embryo from Naef's stage XV. According to Boletzky (2010), the yolk mass passes through the bloodstream into the digestive system to form a nutritive reserve (the inner yolk sac). This inner yolk provides the newly born paralarvae the possibility to obtain the required energy for the first days of life, although octopus paralarvae are able to feed immediately after hatching (Villanueva *et al.* 2002; Iglesias *et al.* 2006; Boletzky 2010). O'Dor *et al.* (1986) and Vidal *et al.* (2002, 2005), working with squid paralarvae showed that the amount of inner yolk mass is proportional to the weight of the embryo and its absorption is related to temperature.

The energy requirements are inferred through oxygen consumption, documented for *Octopus vulgaris* (Parra, Villanueva & Yúfera 2000; Repolho *et al.* 2014) *Octopus maya* (Rosas *et al.* 2009) *Octopus mimus* (Uriarte *et al.* 2012; Zuñiga *et al.* 2013) and *Octopus huttoni* (Higgins, Bates & Lamare 2012). Currently, the only existing parameter to describe yolk values as kcal is specific to *Loligo opalescens* (O'Dor & Wells, 1987). In addition, metabolism would be measured in kcal day^{-1}. Some authors used the yolk weight and kcal of yolk to calculate metabolism (O'Dor *et al.* 1986; Vidal *et al.* 2002, 2005). Unfortunately, there are no yolk calorimetry studies for *O. vulgaris*. For this reason, we used only the yolk weight data as energetic requirements value. In addition, for the first time, we introduce parameters describing the outer yolk density and moisture content for *O. vulgaris* embryos.

The main goal of this research was to analyse how temperature affects the embryos energy demands during embryonic development once completed the process of organogenesis (Naef stage XV) and the transfer and accumulation of inner yolk. Also, internal yolk use by hatched paralarvae subjected to starvation was determined, according to the both temperatures. These results could contribute to the design of experimental rearing processes considering parameters such as the temperature in the first days after hatch (DAH) and in the last stage of embryonic development. In addition, in this study, yolk dry weight was used as an indicator of energy requirements, since it was the unique input of energy available to the paralarvae.

Materials and methods

Experimental design

Eight adult octopuses (six females and two males of 1.1 kg ± 0.2) were captured from the Ría de Vigo (NW Spain) and maintained in a flow-through concrete tank (4.60 m L × 2.10 m W × 1.0 m depth) during 6 months (reaching a mean weight of 3 ± 0.5 kg). The octopuses were fed with fresh mussels (*Mytilus galloprovincialis*), fish (*Merluccius merluccius* and *Sardina pilchardus*) and crustaceans (*Polybius* sp). Inside the tank, several 50 cm long PVC pipes with 18 cm in diameter were placed to serve as dens for females egg laying. Once two females remained within their den without eating, they were individually placed in two smaller tanks (1.0 m L × 1.0 m W) of 0.35 m in a water depth, with a flow-through system. Low light intensities (300 lux) on the water surface were used. The experiment was designed at two temperatures, from the beginning of the spawning, (14°C and 18°C) which are regular mean values from spring until autumn in the Galician Rias (NW Spain). Egg batches were laid in the middle of the PVC pipe on its upper site, so the clusters of eggs remained vertically suspended and enabled the female to maintain constant oxygenation and cleaned the eggs. This was further assisted with gentle water movements produced by the syphon. The water temperature was measured daily. Dissolved oxygen and pH were measured once a week. The same egg clusters were used to describe the embryonic development stages.

For both temperatures, 10 clusters were introduced inside the PVC pipe near the others. To allow clusters to be dragged out of the PVC pipe for collection at each sampling period every 3 or 4 days during embryonic development. Outer yolk and inner yolk were measured throughout this period. Approximately, 100 eggs were used for each temperature treatment.

Experiment 1. Yolk density, moisture and dry weight % of outer yolk and paralarvae moisture content

To obtain density and moisture values of yolk, the outer yolk sac from embryos at Naef stage XVII and XIX was extracted and separated (eggs of three different spawning batches at 14°C and other three at 18°C, $n = 60$). The chorion was cleaved with a scalpel and with two mounted needles embryos were extracted together with the external yolk. The outer yolk was separated from the embryo (Fig. 1) and gently washed in distilled water. The yolk and the membrane were weighed in an ultra-precision scale (0.000001 g) UM3 Mettler (Mettler-Toledo International Inc, Columbus, USA) to obtain wet weight. Dry weight was obtained after 24 h in a dry oven at 80°C.

The yolk density (OYρ), yolk moisture content [OY_M (%)] and the paralarvae moisture (PM) content were calculated using:

$$OY_\rho\ (mg^*mm^{-3}) = OY_{WW}/SV \quad (1)$$

$$OY_M\ (\%) = 100 - ((OY_{DW}/OY_{WW}) * 100) \quad (2)$$

$$P_M\ (\%) = (P_{DW}/P_{WW}) * 100 \quad (3)$$

where the OY_{WW} is the outer yolk wet weight, OY_{DW} is the outer yolk dry weight and SV is the volume of yolk sac. P_{WW} is the paralarvae wet weight and P_{DW} is the paralarvae dry weight.

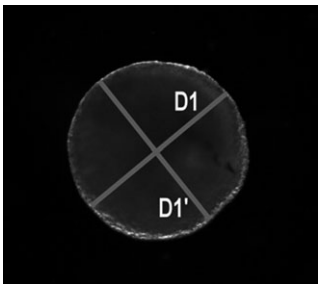

Figure 1 Outer yolk extracted to determine the yolk density and moisture.

Experiment 2. Effect of temperature to yolk consumption and storage during the last stage of development (XV–XX) and yolk consumption during first life stage of paralarvae

Morphometric analysis

Eggs from each treatment were introduced in a Petri plaque with seawater under a magnifying glass Leica MZ8® (Leica Microsystems, Inc., City, IN, USA) and photographed with a Leica IC80®HD camera. Measurements were taken using Leica Application Suite V4 software. The stage of embryonic development was determined according to Naef (1928). Besides the classical Naef XIX stage (with outer yolk sac), an additional stage was included, corresponding to the Naef XIX stage without outer yolk sac (XIX (WOY)), the morphologic characteristics of this new stage of development are the absence of outer yolk and embryos perfectly formed (Fig. 2). To calculate the volume of the outer and inner yolk sacs (SV), sac diameter measures were taken and according the formula:

$$SV = 4/3\pi\ r1 * r2 * r3 \quad (4)$$

where the radius was calculated as S1/2 and S2/2 and S3/2 to obtain $r1$, $r2$ and $r3$ values respectively. S1 and S2 are dorsal measures in length and width and S3 was the lateral view (Fig. 3).

Transfer of outer yolk to the inner yolk sac was evaluated in the form of dry weight. To do so, outer and inner yolk wet weight (Y_{WW}) were calculated to each embryo ($n = 100$) grouped at Naef stage [XV to XIX (WOY)] and a both temperature (14 and 18°C):

$$Y_{WW} = \rho Y * SV \quad (5)$$

where ρY is the density of outer yolk, which was used as value of density for the inner yolk too, (eqn 1) and SV is the volume (eqn 4).

Value of yolk moisture content [Y_M (%)] is 10% (eqn 2) use as correction factor to calculate the yolk dry weight. Value of paralarvae moisture content is 80% [P_M (%), eqn 3] (Table 1).

Outer and inner yolk variations and rate of embryos outer yolk utilization

The differences in embryo yolk dry weight (outer and inner ΔY_{DWs}) between two consecutive Naef stages, from XV to XIX (WOY) at both tempera-

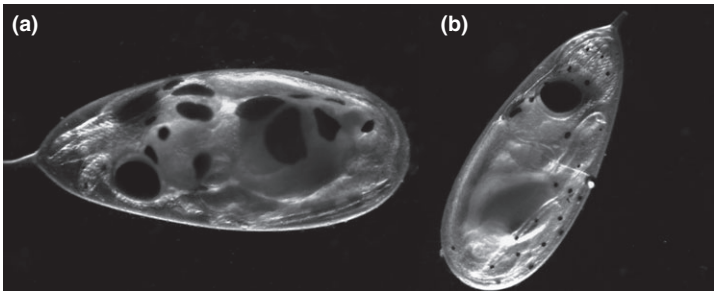

Figure 2 Embryo pre-hatch in the new stage of development described in this study as XIX (WOY), (a) dorsal view, (b) lateral view.

tures were measured according to Vidal *et al.* (2002):

$$\Delta Y_{DWs} = Y_{DWa0} - Y_{DWa} \quad (6)$$

$$Y_{DW} = Y_{DW_0} \exp^{bd} \quad (7)$$

where ΔY_{DWs} is the increment of yolk dry weight (mg) between a particular Naef stage (Y_{DWa}) and the previous one (Y_{DWa0}). Inner and outer yolk of embryos on XV to XIX Naef stage were analysed, in XIX (WOY) embryos on Naef stage the outer yolk value was zero. Y_{DW} is the dry weight (mg), Y_{DWo} is the initial dry weight (mg), b is the body DW (mg) and d is age in days.

Yolk consumption

Embryo yolk consumption from Naef XV until hatching was calculated as the difference between the outer yolk lost. Those data were interpreted as the outer yolk that was consumed by the embryos and stored in the inner yolk sac.

$$\Delta YC = \Delta OY - \Delta IY \quad (8)$$

where ΔYC is the quantity of yolk (mg DW) that was consumed at each embryo grouped at Naef stage at both temperatures, and ΔOY and ΔIY are the dry weight of outer and inner yolk dry weight obtained at each Naef stage respectively.

Table 1 Values of volume, density and moisture of outer yolk of embryos of *Octopus vulgaris* (n = 60)

Outer yolk	Naef stage XVII	Naef stage XIX
Volume (mm^3)	0.31 ± 0.02	0.12 ± 0.01
Wet weight (mg)	0.32 ± 0.02	0.13 ± 0.01
Dry weight (mg)	0.29 ± 0.01	0.11 ± 0.01
Density ($mg*mm^{-3}$)	1.03 ± 0.02	1.03 ± 0.02
Moisture (%)	10.4 ± 3.09	10.3 ± 2.36
DW (%)	89.58 ± 3.09	89.72 ± 2.36

Paralarvae starvation

Twelve hours before the beginning of the experiment all paralarvae were removed from the incubation tank to make sure all newly hatched paralarvae were born at the same time. Newly hatched paralarvae incubated at 18°C (2000 ± 100) during embryonic development, were placed into two different tanks of 100 L using a collector with a 150 μm sieve, with slowly aeration and open flow-through, one at 14°C and other 18°C. We used the same methodology for

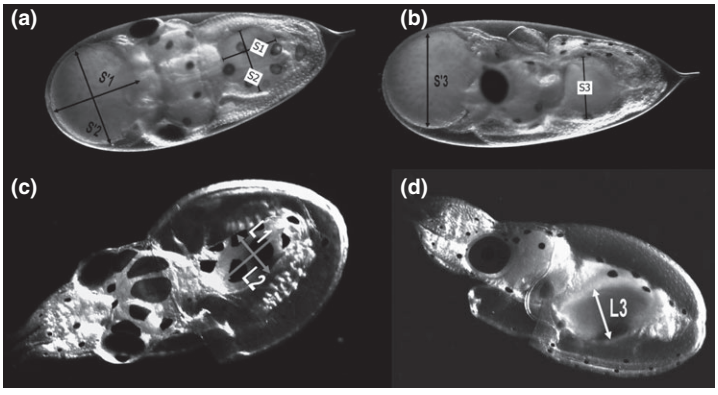

Figure 3 Measurements of external and internal yolk sac diameters in *O. vulgaris* embryos and paralarvae. (a) Embryo dorsal view, (b) embryo lateral view, (c) paralarvae dorsal view, (d) paralarvae lateral view.

the paralarvae that were incubated at 14°C during the embryonic development.

During 4 days all paralarvae for the four treatments remained in starvation. Individual paralarvae dry weights ($n = 80$) and inner yolk measurements ($n = 400$) were recorded daily for the treatments.

Paralarvae inner yolk and inner yolk rate

In the case of paralarvae, the relationship between inner yolk weight and growth rate during 4 days in starvation was calculated using the same methodology to calculate the embryo inner yolk dry weight. Dry weight was used as an indicator of somatic growth expressed as an instantaneous growth rate over their survival time (Forsythe & Van Heukelem 1987).

The exponential function used according to Vidal *et al.* (2005) corresponds to eqn (6). Where, Y_{DW} is the paralarvae inner yolk dry weight (mg), Y_{DWo} is the initial dry weight (mg), b is the instantaneous growth rate in body DW and d is age in days.

Paralarvae dry weight and growth rate

Dry weight of 20 paralarvae per treatment was obtained individually, using small handmade aluminium baskets, after 24 h in a dry oven at 80°C using an ultra-precision scale (0.000001 g) UM3 Mettler. The yolk consumption of paralarvae under starvation at both temperatures was obtained from the values of daily dry weight loss of inner yolk (P_{IYL}) and paralarvae weight loss (P_{DWL}), also were calculated the paralarvae growth rate (SGR%) as the daily growth (t = day):

$$P_{DWL} = P_{DW0} - P_{DWf} \quad (9)$$

$$P_{IYL} = P_{IY0} - P_{IYf} \quad (10)$$

$$SGR\% = ((LnP_{DWf} - LnP_{DW0})/t) * 100 \quad (11)$$

where paralarvae dry weight loss (P_{DWL}) was calculated as the difference between paralarvae dry weight at the beginning of starvation (P_{DW0}) and paralarvae dry weight at the end (P_{DWf}). The same methodology was used to calculate the inner yolk dry weight loss (P_{IYL}) [paralarvae inner yolk dry weight (P_{IY0}) and paralarvae inner yolk final dry weight (P_{IYf})]. The percentage of weight lost at the end of 4 days of starvation was calculated as the inner yolk weight loss for both temperatures. The inner yolk weight and paralarvae weight per day were also determined.

Statistical analyses

Embryos were grouped according to Naef stage, from XV to XIX (WOY) at both temperatures. All data were checked for normal distribution with the one-sample Kolmogorov–Smirnoff test as well as for homogeneity of the variances with the Levene's test and, when necessary, arcsin transformation was performed (Zar 1999). Two treatments were compared with ANCOVA analysis, using the statistical programme STATISTICA 10.0©. Significant differences were considered when $P < 0.05$. When the test was significant, individual means were compared using Tukey's test (Zar 1999).

Results

Embryonic development

Temperature had a great influence on embryonic development time, except for the transition to XV Naef stage until second inversion. Higher temperatures resulted in lower incubation times, with 64 days at 14°C and 40 days at 18°C. The time required to attain the Naef XV stage was 20 days at 18°C and 40 days at 14°C, representing 50% and 62.5% of the total incubation time respectively. In this study, development time between XV and XIX stages (until the second inversion) was 14 days, regardless of temperature. Afterwards, embryos at 18°C and 14°C hatched after 6 and 10 days respectively.

Outer yolk

Outer yolk density and moisture percentage were 1.03 mg*mm^{-1} and 10%, respectively (Table 1). Outer yolk consistently decreased between Naef stages XV to XIX. There were no significant differences (ANCOVA; $F = 6.44$; $P = 0.1579$) in the decrease in outer yolk between Naef stages, both at 14°C and 18°C. Outer yolk dry weight at stage XV was 0.699 ± 0.037 mg and 0.709 ± 0.052 mg at 14°C and 18°C respectively, and at stage XIX 0.150 ± 0.042 mg and 0.155 ± 0.034 mg at

14°C and 18°C respectively. With regard to the embryo outer yolk consumption ratio between Naef stages XV and XIX, the outer yolk consumption ratio decreased −0.15 ± 0.02 mg for each Naef stage at both temperatures. There were no significant differences ($P > 0.05$) in yolk variations between consecutive Naef stages when comparing both temperatures. Furthermore, there is a tendency towards a stable decrease in outer yolk between stages at both temperatures (tendency line is almost parallel to the *X* axis).

Embryo inner yolk

Temperature influenced the amount of the inner yolk accumulated during embryonic development. Significant differences (ANCOVA; $F = 1673.4$ $P = 0.0001$) at all Naef stages from XV to XIX (both with and without outer yolk) were observed between the two temperatures, as well as among stages within the same experimental temperature. Inner yolk was always higher in embryos at 14°C. Inner yolk dry weight was 0.003 ± 0.0006 mg and 0.002 ± 0.0002 mg at stage XV at 14°C and 18°C, respectively, and 0.136 ± 0.011 mg and 0.0.130 ± 0.011 mg at stage XIX at 14°C and 18°C respectively. The sub-stage XIX without outer yolk showed the biggest differences (Fig. 4), being the dry weight of inner yolk of embryos at 14°C (0.213 ± 0.021 mg) significant higher (ANCOVA; $F = 377.14$; $P = 0.0001$) than that of the embryos maintained at 18°C (0.165 ± 0.024 mg).

Use of outer yolk reserves by embryo: consumption and storage

The consumption of outer yolk reserves by the embryos, at both temperatures and from XV to XVIII Naef stages, corresponded to the 80 ± 10% of their outer yolk dry weight. The remaining yolk (20 ± 10%) was stored as inner yolk (Fig. 5). Nevertheless, at Naef stage XIX an important difference on the consumption of outer yolk was observed; in this case, at 14°C approximately 50% of the outer yolk was consumed and 50% was transferred to the inner yolk sac. Contrary, at 18°C, yolk consumption remains as in previous (80 ± 10%) to XV until XVIII Naef stages (Fig. 5).

Paralarvae inner yolk dry weight ratio

Two types of experiments were conducted; in the first one, we used paralarvae incubated at 18°C and transferred at 14 and 18°C in starvation. Inner yolk weight was higher at 14°C during the first 24 h after hatching, being of 0.101 ± 0.05 and 0.08 ± 0.05 mg DW at 14°C and 18°C respectively (Fig. 6). There were significant differences in the paralarvae inner yolk weight at first 24 h (ANCOVA; $P = 0.007$) and at 4 days after hatching (ANCOVA; $F = 8.76$; $P = 0.009$). During the first day, paralarvae at 14°C lost the 7% of inner yolk dry weight while those at 18°C lost the 25%. After 4 days of starvation, paralarvae at 14°C lost 50% of the yolk reserves (0.055 ± 0.01 mg DW) compared to 67% at 18°C (0.036 ± 0.001 mg DW).

To conduct the second experiment we used paralarvae incubated at 14°C and transferred at 14 and 18°C starvation conditions. Inner yolk weight was higher at 14°C during the first 24 h after hatching, being of 0.118 ± 0.03 and 0.099 ± 0.02 mg of inner DW at 14°C and 18°C respectively (Fig. 6). There were significant differences) in the paralarvae inner yolk weight at first 24 h (ANCOVA; $P = 0.0007$) and 4 days after hatching (ANCOVA; $P = 0.0008$). During the first day,

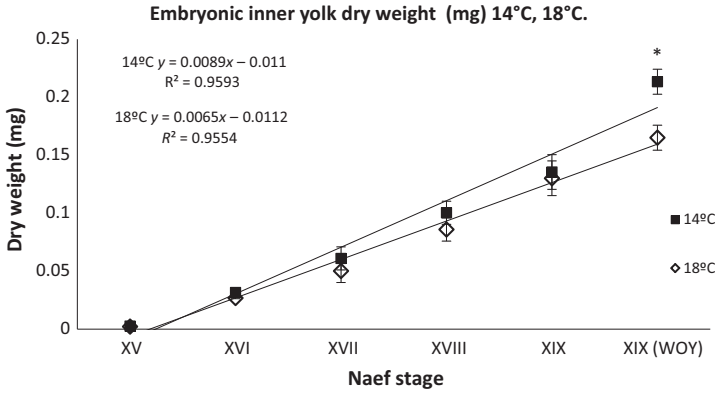

Figure 4 Inner yolk dry weight (mg) of *O. vulgaris* embryos at 14°C and 18°C. Bars are standard deviations and "*" indicates significant ($P < 0.05$) differences at the two temperatures.

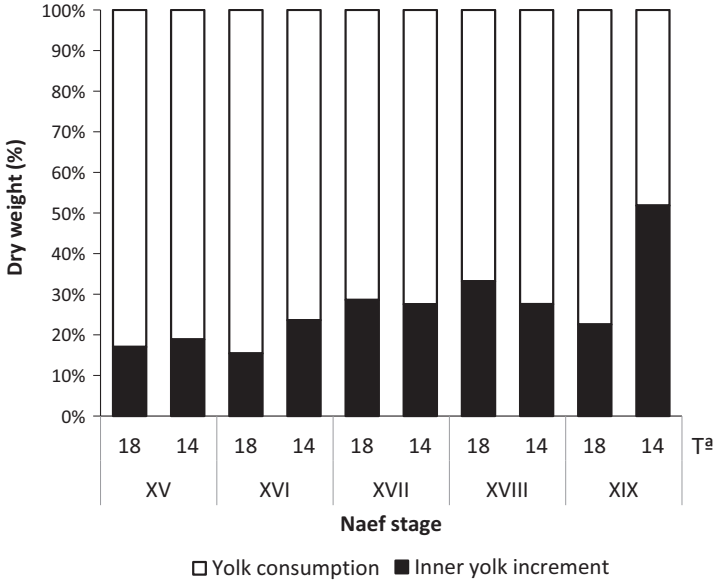

Figure 5 Percentage of outer yolk used and inner yolk increment between consecutive Naef stages for *O. vulgaris* embryos at 14°C and 18°C.

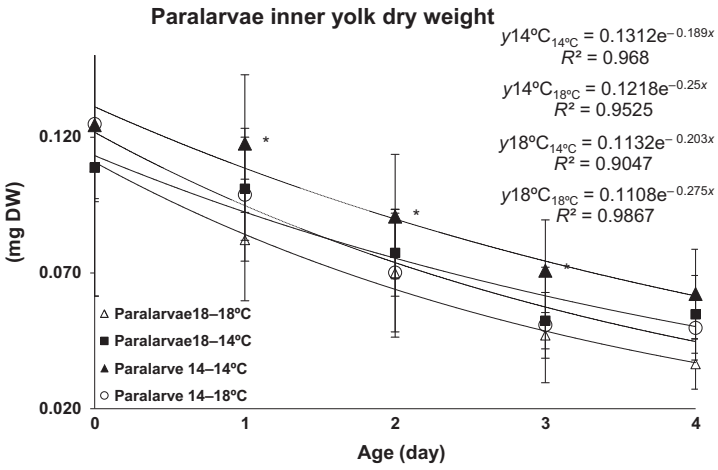

Figure 6 Inner yolk dry weight (mg DW) for *O. vulgaris* unfed paralarvae during 4 days, at 14°C and 18°C. Bars represent standard deviations and '*' indicates significant ($p<0.05$) differences at both temperatures.

paralarvae at 14°C lost the 5% of inner yolk dry weight while those at 18°C lost the 21%. After 4 days of starvation, paralarvae at 14°C lost the 50% of the yolk reserves (0.062 ± 0.02 mg IY_{DW}) and the 60% at 18°C (0.049 ± 0.01 mg IY_{DW}).

There were significant differences (ANCOVA; $P = 0.0004$) on inner yolk consumption per day between paralarvae in starvation at same temperature and embryos incubated at both temperatures (14 and 18°C). The inner yolk consumption per day for unfed paralarvae at 18°C incubated at 14°C was higher than the paralarvae which were incubated at 18°C, being 0.015 mg day^{-1}, 0.009 mg day^{-1} respectively. Therefore, the paralarvae that were incubated at low temperature, at the end of 4 days, had higher inner yolk reserves than those incubated at 18°C. Paralarvae incubated at 14°C and 18°C and both maintained at 14°C in starvation showed significant differences in yolk consumption.

Paralarvae weight and growth rate

Paralarvae had negative growth throughout the 4 days of starvation (Fig. 7). Loss of weight was exponential throughout this period, the growth standard rate per day (ANCOVA, $F = 1377.9$; $P = 0.006$) was −4.42 mg day^{-1} at 14°C and −6.52 mg day^{-1} at 18°C.

Paralarvae hatched and maintained at 18°C were lighter at day 3 and 4 than those at 14°C.

These paralarvae lost 10.68% and 26.11% of dry weight by day 2 and 4 respectively. During the first 2 days of starvation, there were no significant differences (ANCOVA; $P = 0.005$) in paralarvae dry weight to paralarvae incubated at 18°C. Paralarvae that were hatched at 18°C but were maintained in starvation at 14°C lost 9.72% and 17.68% of their body weight by day 2 and 4 respectively.

Paralarvae hatched and maintained at 14°C lost 2.16% and 12.37% of their body weight by days 2 and 4 respectively. Paralarvae hatched at 14°C and maintained at 18°C lost 11.45% and 20.41% of dry weight by day 2 and 4 respectively. There were significant differences (ANCOVA; $F = 12222.6$, $P = 0.0001$) during all days of starvation except at the day 3, in paralarvae dry weight to paralarvae incubated at 18°C.

Discussion

Temperature greatly influenced embryonic development time, which was 64 and 40 days at 14°C and 18°C respectively. Moxica, Otero, Iglesias and Sánchez (2001) reported 38 days at 18.7°C. Embryo development time was markedly different until Naef stage XV, being of 20 days at 18°C and 40 days at 14°C. It is noteworthy that after this stage (when inner yolk appears) and until second inversion, the development time was independent from temperature (14 days both at 14°C and 18°C). This fact could be explained because the embryos rate of outer yolk consumption was similar (80 ± 10%) at both temperatures until stage XIX, volume transferred each Naef stage used part to supply yours energetic requirements and remainder for storage in the inner yolk sac.

At higher temperatures, a reduction in the inner yolk content at hatching was expected, due to increased energy expended by the embryo (Bermudes & Ritar 1999). When the basic processes of organogenesis and the progressive contraction of the embryo cap have ended by stage XV, the small inner yolk sac begins to increase in size, due to a slow transfer of outer yolk (Fioroni & Boletzky 1990; Boletzky 2002). The pace of this transfer, which finally inverts the respective volumes of the two yolk compartments (outer and inner yolk sac), seems influenced by the temperature at which the embryo develops after XV until XIX (Bouchaud & Daguzan 1990; Bouchaud & Galois 1990). Inner yolk variations between Naef stages XV and XIX for embryos developing at 14°C and 18°C show a higher inner yolk accumulation in embryos at 14°C than at 18°C. The highest value (0.078 mg) was observed in embryos at 14°C, when outer yolk was absent [XIX (WOY), Naef stage].

This fact was supported by many studies that have shown that inner yolk volume at hatching is inversely correlated with incubation temperature in many fish species (Canino 1994; Bermudes & Ritar 1999) and cephalopods such as the cuttlefish, *S. oficinalis* (Bouchaud 1991), the squid *L. opalescens* (Vidal *et al.* 2002) and *O. mimus* (Uriarte *et al.* 2012). This was also true in this study, when comparing inneryolk weight at the two temperatures. Just before hatching embryos developing at 14°C had more inner yolk than those developed at 18°C. These differences are in agreement with the results obtained by O'Dor *et al.* (1986) and Vidal *et al.* (2002, 2005) for squid paralarvae. These authors reported that at lower temperatures, the accumulation of inner yolk was higher and the degree of energy consumption was lower.

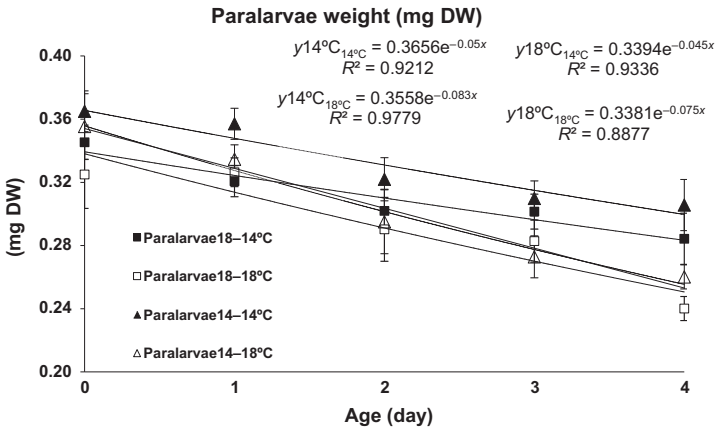

Figure 7 Dry weight of *O. vulgaris* paralarvae during 4 days of starvation, at 14°C and 18°C. Bars are standard deviations.

Outer yolk utilization between consecutive Naef stages was similar (80 ± 10%) at both 14°C and 18°C between stages XV and XVIII. Finally, between stage XIX and hatching (which lasted for 6 days at 18°C and 10 days at 14°C), the proportion of outer yolk incorporated to the inner sac at 14°C increased from 20% to 50%, while at 18°C it remained at 20%. This explains the higher inner yolk content in newly hatched paralarvae at 14°C. During this last stage, embryos accumulate all yolk reserves within their inner sack. In the new stage described in this study [XIX (WOY)] the embryo is ready to hatch, but differences on time exists at both temperatures, this may be motivated to regulation by perivitelline fluid and the hatching gland. In addition, the movement of string by the mother may be a mechanism to regulate the time of hatch (Villanueva & Norman 2008).

For *S. officinalis*, lower temperatures increase embryonic development time and deliver larger hatchlings (Choe 1966; Bouchaud & Daguzan 1990; Bouchaud 1991). Nevertheless, during this study, at the end of embryonic development newly hatched paralarvae had similar weight at 14°C and 18°C.

Paralarvae at 18°C have shown faster inner yolk consumption than paralarvae at 14°C, suggesting that at high temperature paralarvae will complete the development faster than those maintained at 14°C. Forsythe (1993) noted the tendency among shallow-water cephalopods to spawn in colder but increasing temperatures (spring), so that hatchlings would be exposed to continually increasing temperatures for the first few months of life when exponential growth is observed. Also in a recent study, Semmens, Doubleday, Hoyle and Pecl (2011) observed that *Octopus pallidus* dedicated proportionally more energy to gonad growth when exposed at low temperatures than exposed at high temperatures, suggesting that a similar mechanism that observed by Forsythe (1993) could be modulating reproductive performance in *O. pallidus*. According to Vidal *et al.* (2002) at least in the range 12–16°C, *L. opalescens* paralarvae inner yolk weight was proportional to the body mass, representing about 10–15% of the body wet weight. During this study, inner yolk also represented about 10% of *O. vulgaris* paralarvae of dry weight body mass, both at 14°C and 18°C. Slopes of the exponential fraction of yolk utilization showed higher paralarvae yolk ratios for starved hatchlings at the higher temperature. These figures are in agreement with the fact that all energy requirements are covered exclusively by yolk consumption and there is no contribution of external feed. According to Vidal *et al.* (2002), with daily exponential yolk absorption rates of 43%, starved hatchlings at 12°C entered into an energy deficit 3 days after hatching. This value increased to 50% at 16°C. Paralarvae inner yolk utilization was also always higher at 18°C in this study. Yolk utilization was of 8% at 14°C and 25% at 18°C after the first 24 h. At the end of 3 days in starvation, paralarvae had used 50% of the inner yolk at 14°C and 60% at 18°C. To balance this deficit, paralarvae must quickly obtain an extra source of energy through external feeding.

Paralarvae maintained in starvation at 18°C lost between 10.68% and 26.11% of their body weight by day 2 and 4 respectively. These results are similar to those obtained by Villanueva, Riba, Ruíz-Capillas, González and Baeta (2004), where *O. vulgaris* paralarvae lost between 16% and 28% of dry weight after 2 and 4 days, respectively at 20°C. Observations under experimental conditions show that well-developed, non-premature hatched *O. vulgaris* paralarvae start feeding during the first 24 h after hatching (Villanueva *et al.* 2002; Iglesias *et al.* 2006) and that the presence of an inner yolk sac does not apparently interfere with any organ functioning (Boletzky 2010). According to Chen, Dykhuizenv, Hodge and Gilly (1996) and Villanueva *et al.* (2004) the process of prey capture by early hatched paralarvae is an acquired behaviour. Although during early stages paralarvae feed on prey with slow movements, it seems essential, as happens in squid paralarvae (Chen *et al.* 1996), that during the first few days after hatching they remain in contact with live preys to learn the feeding behaviour that will be used in the following development days. During the first 2 days after hatching, a high percentage of attacks are unsuccessful (Iglesias *et al.* 2006), and paralarvae must improve their attack strategy to be more efficient in obtaining prey. This feeding behaviour consumes energy and paralarvae can obtain it by endotrophic feeding of the yolk reserves until learn more effective attacks. Therefore, they can combine endotrophic with exotrophic feeding. When these reserves are depleted, the weight loss is higher because paralarvae obtain their energy from the muscle. Therefore, lower temperatures would increase the "learning time" available for paralarvae to become more effective in the capture

of prey. Contrary, at higher temperatures there would be less time available to optimize prey capture techniques, and could accelerate and increase mortality.

Based on these results, it would be advisable to design experiments in which the early stages of embryonic development occur at higher temperatures respecting the threshold of maximum development temperature for cephalopods. Embryonic development will then occur in a shorter period. However, when stage XV is reached it could be recommended to reduce the temperature gradually to enhance inner yolk accumulation. With recently hatched paralarvae, lower temperatures could be beneficial, since paralarvae will have greater volume of inner yolk sac, and weight loss occurs at slower pace until they acquire sufficient experience in prey capture. According to Boletzky (2002), the volume of yolk remaining in the inner sac at hatching is one of the major factors influencing the initial feeding conditions of the hatchling. This initial period of learning at lower temperatures would imply more time to further refine the process of prey attacks and paralarvae would be more effective in obtaining external feed.

In any case, more experiments describing processes of exogenous and endogenous feeding are necessary since it is essential to consider aspects related to a gradual adaptation to the hunting procedures or techniques. Other aspects to investigate in the phase of exogenous feeding of *O. vulgaris* paralarvae are the selection of prey and the development of the sensorial and locomotion system.

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